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AWARD NUMBER: DAMD17-01-1-0352

TITLE: Neural Protein Synuclein (SNCG) in Breast Cancer Progression

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REPORT DATE: August 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*
01-08-2005

2. REPORT TYPE
Annual Summary

3. DATES COVERED *(From - To)*
1 Jul 2004 – 31 Jul 2005

4. TITLE AND SUBTITLE

Neural Protein Synuclein (SNCG) in Breast Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0352

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Long Island Jewish Medical Center
Lake Success, New York 11042

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Synucleins are emerging as central players in the formation of pathologically insoluble deposits characteristic of neurodegenerative diseases. However, synuclein y (SNCG), previously identified as a breast ctancer specific gene (BCSGI), is also highly associated with breast cancer progression. Using transgenic mouse model, we demonstrated a role of SNCG in induction of highly proliferative pregnancy-like phenotype of niammary epithelial cells, branching morphology, and mammary hyperplasia. SNCG participated in the heat slhock protein-based multiprotein chaperone complex for steroid receptor signaling. Expression of SNCG in mammary epithelium resulted in a significant stimulation of **ERa** transcriptional activity. SNCG-induced mammary hyperplasia can be effectively blocked by antiestrogen and ovariectomy, indicating that the induced hyperplasia is mediated by **ERa** signaling and requires estrogen stimulation. These data indicate the chaperone activity of SNCG on stimulation of steroid receptor signaling in mammary gland and, thus induces mammary hyperplasia and contributes to the hormonal impact on mammary tumorigenesis.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

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A. INTRODUCTION

We have previously reported the isolation of differentially expressed genes in the cDNA libraries from normal breast and infiltrating breast cancer using differential cDNA sequencing approach (1-3). Of many putative differentially expressed genes, a breast cancer specific gene BCSG1 was identified as a putative breast cancer marker. This gene was highly expressed in the advanced breast cancer cDNA library but not in a normal breast cDNA library (1). Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes. Rather, there is extensive sequence homology to neural protein synuclein. Subsequent to the isolation of BCSG1, synuclein γ (SNCG) (4) and persyn (5) were independently cloned from a brain genomic library and a brain cDNA library. The sequences of these two brain proteins were found to be identical to BCSG1. Thus, the previously identified BCSG1 has also been named as SNCG and is considered to be the third member of the synuclein family (6).

Synucleins are a family of small proteins consisting of 3 known members, synuclein α (SNCA), synuclein β (SNCB), and synuclein γ (SNCG). Synucleins have been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Mutations in SNCA is genetically linked to several independent familial cases of PD (7). More importantly, wild type of SNCA is the major component of Lewy bodies in sporadic PD and in a subtype of AD known as Lewy body variant AD (8-9). SNCA peptide known as non-amyloid component of plaques has been implicated in amyloidogenesis in AD (10-11). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy bodies cases (12-13). Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, they have also been implicated in non-neural diseases, particularly in the hormone responsive cancers of breast and ovary (1-2,14-21).

Being identified as a breast cancer specific gene, SNCG expression in breast follows a stage-specific manner (1). Overall SNCG mRNA expression was detectable in 39% of breast cancers. However, 79% of stage III/IV breast cancers were positive for SNCG expression, while only 15% of stage I/II breast cancers were positive for SNCG expression. In contrast, the expression of SNCG was undetectable in all benign breast lesions (17). The expression of SNCG was strongly correlated to the stage of breast cancer. Overexpression of SNCG in breast cancer cells led to a significant increase in cell motility and invasiveness *in vitro* and a profound augmentation of metastasis *in vivo* (14), resistance to chemotherapeutic drug-induced apoptosis (22), and accelerated rate of chromosomal instability (21). Overexpression of synucleins, especially SNCG, also correlated with ovarian cancer development (4,18,23). While synucleins (α, β, and γ) expression was not detectable in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express either SNCG or SNCB, and 42% (19 of 45) expressed all three synucleins (α, β, and γ) simultaneously (18). The involvement of SNCG in hormone responsive cancers of breast and ovary promoted us to explore the potential role of SNCG in cellular response to estrogen. Previously, we demonstrated a function of SNCG to stimulate hormone dependent growth of breast cancer cells both in vitro and in nude mice (16). In the present study, we evaluated the *in vivo* function of SNCG in mammary gland development and mammary pathogenesis. The results indicated that SNCG, acting as a chaperone protein for ERα and PR, stimulates steroid receptor signaling in mammary epithelial cells and mammary gland, induces a highly proliferative pregnancy-like phenotype, and mammary hyperplasia.

B. RESEARCH REPORT

SA1. Does SNCG overexpression in MMTV/SNCG transgenic mice alter mammary gland development? Finished.

B1. Effects of expression of SNCG transgene on mammary gland development. We generated five SNCG transgenic lines and picked up one stable line named MMS1, which expressed relative high level of SNCG. Transgene expression in the mammary gland was assayed by RT-PCR and Western analyses (Fig. 1). In the mammary glands from virgin MMS1 mouse, the expression of the transgene was detected by RT-PCR using the primers specific for human SNCG (Fig. 1A). No signal was detected in the mRNA isolated from mammary glands of virgin wild-type females. Consistent with the transgene mRNA expression, while no SNCG protein was detected in the gland from the virgin control females, SNCG protein was highly

expressed in the mammary gland from MMS1 mouse (Fig. 1B). Both MMS1 mice and mice from other transgenic lines developed normally compared with their nontransgenic littermates.

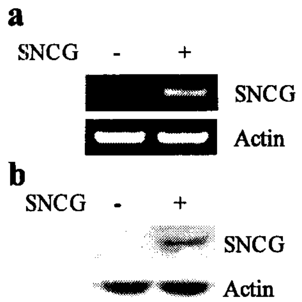

Fig. 1. SNCG transgene expression in control and homozygous transgenic lines. Twelve-week old virgin MMS1 and age matched control virgin mouse were sacrificed and the inguinal mammary glands were removed. The left gland was subjected to RNA isolation and RT-PCR analysis and the right gland was subjected protein isolation and Western analysis. (**a**). RT-PCR analysis of SNCG using primers within SNCG cording sequence. The integrity and the loading control of the RNA samples were ascertained by actin expression (**b**). Western analysis of SNCG protein and actin expression. Western blot using the specific anti-SNCG antibody was carried out on 50 µg of loaded cellular protein.

The effect of transgene expression on mammary gland development was assayed by morphological analyses of ductal elongation and appearance of a branching morphogenesis. While the mammary gland development starts at about 3-week old in wild-type mice with ductal elongation, development of branching structure and functional differentiation starts at the onset of pregnancy with the expansion of secretory lobular-alveolar architecture. Whole mount preparations of the mammary glands starting at 4-week to 8-week from virgin wild type and virgin transgenic mice were examined to determine the effect of SNCG on early mammary gland development. Fig. 2 shows a representative mammary gland analysis of 7-week old transgenic mouse vs. wild-type control littermate. Mammary ducts in the transgenic virgin (Fig. 2B) as well as in the control virgin littermate (Fig. 2A) passed the typical ½ length of the inguinal gland, completely filled with the ducts, and appeared normal. Similar ductal developments were also observed at different time points, indicating that expression of the transgene did not alter the ductal outgrowth during the early mammary gland development. However, an alternation in the developmental pattern of the branching points of ducts in transgenic virgin mouse was observed compared with the control littermate. While the limited branching was developed in the wild-type gland (Fig. 2A), transgenic gland exhibited a multiplicity of branching (Fig. 2B).

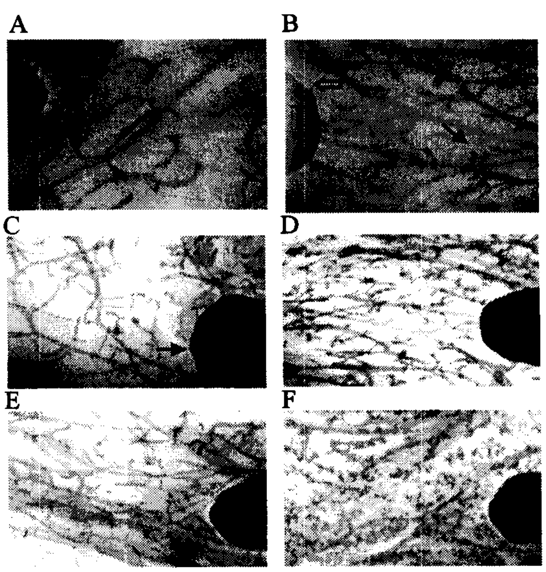

Fig. 2. Whole mount histological analysis of mammary glands from virgin SNCG transgenic mice and wild-type littermates. Control (A, C, E) and age-matched SNCG transgenic mice (B, D, F) were sacrificed, the right inguinal gland was removed and subjected to whole mount gland fix, defat, and staining. A-B, 7-week old mice. Open arrows indicate the inguinal lymph nodes. A solid arrow indicates branching morphology in the transgenic mouse. C-D, 3-month old mice. An arrow indicates the inguinal lymph node. E-F, 5-month old mice. Glands from transgenic mice (D and F) showed an extensive branching morphology.

B2. Induction of highly proliferative pregnancy-like phenotype proliferation and gland hyperplasia. To further confirm our observation of induced branching morphology in SNCG transgenic mouse, we extended the whole mount mammary gland analysis starting at 2-month to 5-month from virgin wild type and virgin transgenic mice to determine the effect of SNCG on development of proliferative branching morphology. Two mice were examined for each time point and data in Fig. 2C-F shows a representative mammary gland analysis of 3-month and 5-month old transgenic mouse vs. age-matched wild-type control littermate. A significant alternation in the developmental pattern of the branching points of ducts in transgenic virgin mice was observed compared with the control littermate. While the limited branching was developed in the wild-type gland (Fig. 2C & E), transgenic gland at 3-month exhibited an extensive

branching morphology (Fig. 2D), indicating proliferation of the cells in the end bud. A robust increase in the branching structure at terminal end was observed in the gland from 5-month virgin transgenic mouse (Fig. 2F), a phenotype similar to the stage of pregnancy.

The increased branching in the transgenic mice suggests a potential effect of SNCG on induction of proliferation of mammary epithelial cells in the end bud. We analyzed the effect of SNCG on mammary epithelial cell proliferation in the transgenic mouse. We measured the percentage of cycling cells determined by BrdU incorporation in three 3-month old SNCG transgenic mice and the three age-matched control mice. While the average percent of labeled nuclei in control mice was 7.1%, expression of SNCG significantly stimulated cell proliferation with a 42% of nuclei labeled cells (**Table 1**).

	Average BrdU labeling (%)	
Age	Control	Transgenic
3-month	7.1 ± 2.2	42 ± 5.1

Table 1. Stimulation of proliferation of mammary cells by SNCG. BrdU was injected i.p. at a dosage of 75 mg/kg into wild type and transgenic mice. After 2 h, the mice are killed, and the right third inguinal glands are removed and processed for BrdU analysis. Tissues are fixed, processed, and sectioned. BrdU is detected using a 1:400 dilution of a rat monoclonal antibody specific for BrdU (Accurate Chemicals) and avidin-biotin complex immunohistochemistry. The percentage of labeled nuclei is determined by randomly counting 500 epithelial cells in 5 fields (40X) with three observers. Numbers are means ± SD of three mice.

Histological evaluation of H & E stained mammary sections also confirmed the presence of multiple branching structure in the gland from the virgin transgenic mice. There were no morphological differences observed in the younger (4-6-week old) transgenic mice compared to the age-matched control mice. However, starting at 10-week old, a significantly different morphology was observed in the transgenic mice vs. the control mice. As shown in Fig. 3, whereas no branching structures were present in the 3-month old control virgin mouse, indicating no proliferation of end bud (Fig. 3A), a branching morphology (Fig. 3B-C) was observed in the transgenic mice, indicating a highly proliferative capability of cells in the terminal end buds. A representative robust branching development was observed in a 5-month old transgenic mouse and illustrated in Fig. 3D. Histological analysis also revealed the presence of mammary hyperplasia in the transgenic mouse. While a normal mammary gland has a single layer of epithelial cells (Fig. 4A), gland from transgenic mouse displayed diffuse acinar hyperplasia (Fig. 4B). No carcinoma was observed in the virgin transgenic mice up to 1 year.

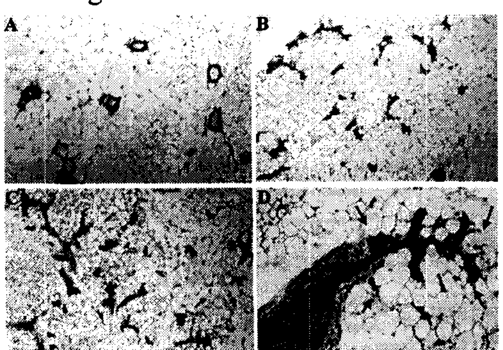

Fig. 3. Histological analysis of mammary gland. Whole inguinal glands were isolated from 3-month (A & B) and 5-month (C & D) virgin mice. All sections were stained with hematoxylin and eosin for histological analysis. A, control mouse. B-D, transgenic mice. A-C, 10 x 10. D, 10 x 40. The proliferation of the cells in end bud or multiple budding was clearly visible in the transgenic glands with a significant increase from the gland in 3-month (**B**) to the gland in 5-month mouse (**C**). A higher magnification (**D**) shows a very organized end bud proliferation and branching (arrows indicated).

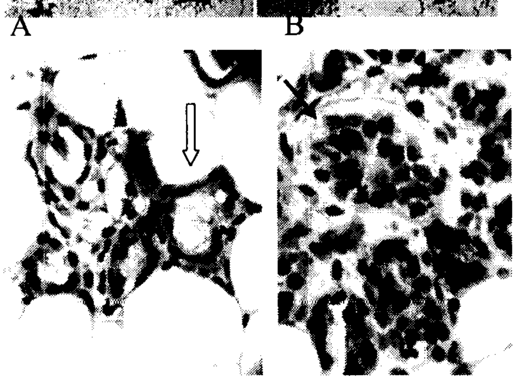

Fig. 4. Induction of mammary hyperplasia in SNCG transgenic mouse. A 5-month control mouse (A) and age-matched transgenic virgin mouse (B) were pre-injected i.p. with BrdU at a dosage of 75 mg/kg as described in Table 1. Inguinal glands were isolated and sections were subjected to both hematoxylin stain for histological analysis and to immunohistochemical stain with rat monoclonal antibody specific for BrdU for reviewing labeled nuclei. Nuclei labeled with brown color indicate proliferating cells. Open arrow indicates normal mammary gland. Solid arrow indicates mammary hyperplasia in the transgenic mouse.

B3. Stimulation of ERα signaling in transgenic mammary gland. We next investigated the effect of SNCG on ERα signaling in mammary gland, which was measured by analysis of ER-mediated transcriptional activity on E_2-regulated genes of PS2 and Cathepsin D (Cat-D). Fig. 5 shows a representative real time RT-PCR analysis of ERα, PS2, and Cat-D mRNA expression in three virgin control mice and three age-matched virgin transgenic mice. While basal levels of PS2 and Cat-D were detected in control mice, forced expression of SNCG in virgin mammary glands significantly enhanced PS2 and Cat-D expression, resulting in an average 3.4-fold (Fig. 5B) and 4.8-fold (Fig. 5C) increase over control mice, respectively. There was no significant change in ERα expression in control vs. transgenic mice (Fig. 5A), indicating that the increased PS2 and Cat-D expression in the transgenic mice is mediated by enhanced ER signaling but not due to the alternation of ER levels. To avoid the different *in vivo* estrogenic environments due to the different hormonal cycling of mice, we used whole mammary organ culture to study whether E2 can differentially regulate Cat-D expression in glands from control and transgenic mice. In this *ex vivo* model, the glands from virgin mice were cultured for 8 days to deplete the endogenous hormones before the E2 treatment. Expression of SNCG was clearly detectable in the glands from transgenic mouse in this short-term organ culture (Fig. 6A). Consistent with the observed stimulation of Cat-D in the transgenic mice, expression of Cat-D was significantly increased in E2 treated transgenic gland, resulting in a 5.4-fold increase over the E2 treated control gland (Fig. 6B).

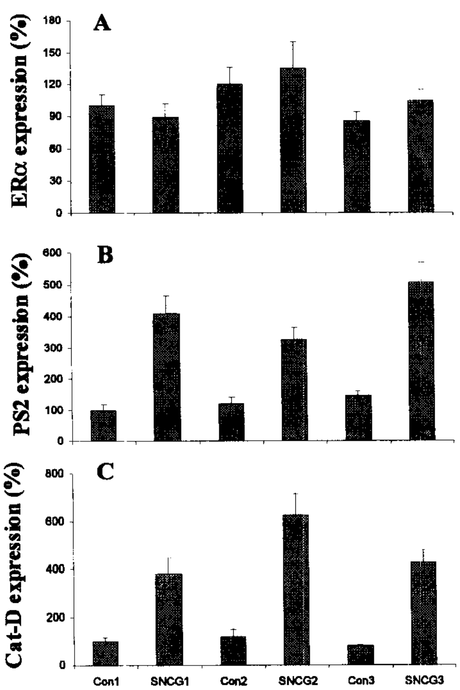

Fig. 5. Quantitative RT-PCR analysis of ERα, PS2, and Cat-D expression. Inguinal mammary glands were isolated from age-matched virgin control and SNCG mice. Mice from Con1 and SNCG1 were 13-weeks old. Mice from Con2 and SNCG2 were 16-week old. Mice from Con3 and SNCG3 are 18-week old. RNAs were isolated and subjected to real time PCR analysis using the TaqMan PCR core reagent kit (Applied Biosystems). Relative expressions of mouse ERα, PS2, and Cat-D gene in the mammary glands from SNCG mice were calculated in comparison to that from Con1 mouse, which was taken as 100% and regarded as control. All the other values were expressed as a percentage of the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate RNA samples.

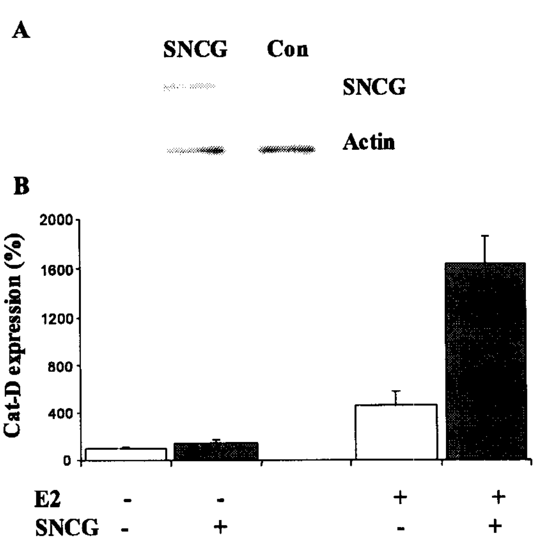

Fig. 6. SNCG stimulated ERα transcriptional activity in mammary organ culture. A pair of inguinal mammary glands from a 14-week virgin control as well as transgenic mouse were isolated and cultured for 8 days in the E2 free mammary organ culture as described in Methods. Glands were treated with or without 10 nM E2 for 24 hours, protein and RNA were isolated. **A**, a Western analysis of SNCG expression in combined control and transgenic glands from both non-treated and E2-treated gland. Expression of SNCG was maintained during short-term organ culture. **B**, a quantitative RT-PCR analysis of Cat-D expression. Relative expression of mouse Cat-D gene in the mammary glands treated with E2 was calculated in comparison to that from control non-treated gland. The gene expression in the control non-treated gland was taken as 100% and regarded as control. All other values were presented as percentage over the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate samples.

SA2. Does overexpression of SNCG in the transgenic mice enhance breast cancer progression? Finished.

Expression of SNCG in mammary gland exacerbates EbB-2-induced mammary tumor development and transactivates ErbB-2. We developed transgenic mice that coexpress both ErbB-2 and SNCG in the mammary epithelium by crossbreeding our MMTV/SNCG transgenic mice with EerB-2 transgenic mice. The effect of SNCG signaling on ErbB-2 induced mammary tumor was investigated in SNCG/ErbB-2 bi-transgenic mice. While expression of SNCG in mammary epithelium induces mammary hyperplasia, MMTV/SNCG transgenic mice fail to develop mammary tumor. Previous studies have shown that ErbB-2 transgenic mice develop multifocal metastatic mammary tumors at ~6 months of age (37). Expression of SNCG in mammary gland significantly stimulated ErbB-2-induced mammary tumorigenesis (**Table 2**). The results reveal that bitransgenics show significantly reduced latency of palpable mammary tumor formation with 50% of the animals showing tumor formation at 121 days as opposed to 189 days for ErbB-2 mice. Tumor growth was also significantly stimulated. At day 250, the average tumor size of bitransgenics was 2.6-fold of that of ErbB-2 mice. There was a slight increase in tumor incidence in bitransgenics, but it was not significant compared with ErbB-2 mice. These data indicate that SNCG exacerbates ErbB-2-induced mammary tumors, suggesting that SNCG may activate ErbB-2 signaling. As shown in **Fig.1**, expression of SNCG significantly increased ErbB-2 phosphorylation and activated its downstream Akt signaling pathways in the rumors from ErbB-2/SNCG bitransgenic mice.

Table 2. Expression of SNCG exacerbates ErbB-2-induced mammary tumor development. The effect of SNCG on ErbB-2-mediated tumorigenesis was measured by tumor incidence (percentage of mice developing tumors at 10-month period), tumor latency T50 (day with 50% of the animals showing tumor formation), and tumor size at the day 250. There were 15 mice analyzed for each group. Statistical comparisons for both T50 and tumor size in SNCG/ErbB-2 mice relative to ErbB-2 mice indicate $p < 0.01$. Statistical comparison for tumor incidence in SNCG/ErbB-2 mice relative to ErbB-2 mice indicate $p > 0.05$.

Experimental Group Genotypes	Tumor incidence Tumor/Total (%)	T50 (Days)	Tumor Size (mm^3) Day 250
SNCG	N/A	N/A	N/A
ErbB-2	58%	189	1031±220
SNCG/ErbB-2	64%	121	2641±421

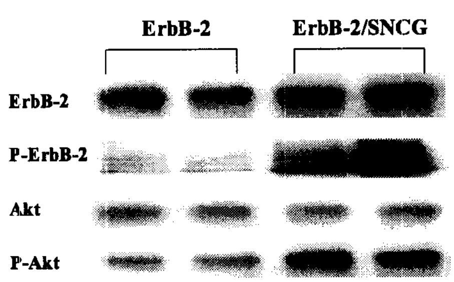

Fig. 7. SNCG transactivates ErbB-2 and Akt in bitransgenic mice. Western analysis of ErbB-2 and Akt phosphorylation in two mammary tumors from ErbB-2 transgenic mice and two tumors from ErbB-2/SNCG bitransgenic mice. Activated ErbB-2 and Akt were normalized with non-phosphorylated ErbB-2 and Akt.

C. KEY RESEARCH ACCOMPLISHMENT (CANCER RES., 63: 3899-3903, 2003; 64: 4539-4546, 2004; Oncogene, in press)

A notable finding in this study is that SNCG, previously identified as a breast cancer specific gene 1 (BCSG1), strongly stimulated the ligand-dependent transcriptional activity of estrogen receptor-α (ER-α) in breast cancer cells as well as in mammary gland.

1. Augmentation of SNCG expression stimulated transcriptional activity of ER-α, whereas compromising endogenous SNCG expression suppressed ER-α signaling. The SNCG-stimulated ER-α signaling was demonstrated in three different cell systems including ER-α-positive and SNCG-negative MCF-7 cells, ER-α-positive and SNCG-positive T47D cells, and SNCG-negative and ER-α-negative MDA-MB-435 cells. The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation

of the ligand-dependent cell growth. While overexpresssion of SNCG stimulated the ligand-dependent cell proliferation, suppression of endogenous SNCG expression significantly inhibited cell growth in response to estrogen. The stimulatory effect of SNCG on ERα-regulated gene expression and cell growth can be effectively inhibited by antiestrogens.

2. SNCG, acting as a chaperone protein, participates in Hsp-based multiprotein chaperon complex for steroid receptors, induced mammary hyperplasia, which is mediated by its stimulation of ligand-dependent transcriptional activity of ERα in mammary gland. Expression of SNCG in mammary gland in the virgin mouse greatly stimulated the proliferation of the mammary cells at end bud and resulted in a robust morphological branching, a phenotype similar to the pregnancy-induced proliferation. The identification of steroid receptors of ERα as molecular target for one of the actions of SNCG on the hormone-dependent growth of mammary gland suggest a critical role of SNCG on the pathogenesis of mammary tumors.
3. Expression of SNCG in mammary gland exacerbates EbB-2-induced mammary tumor development and transactivates ErbB-2.

D. CONCLUSIONS

1. Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, synucleins have also been implicated in non-neural diseases particularly in the hormone-responsive cancers of breast and ovary. SNCG was first identified by differential cDNA sequencing as a breast cancer specific gene, which was expressed abundantly in metastatic breast cancer cDNA library but scarcely in normal breast cDNA library. SNCG expression is highly associated breast cancer and ovarian cancer progression. In addition, overexpression of SNCG in breast cancer cells significantly stimulated cell growth in vitro and tumor metastasis *in vivo*. However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated ER-α as one of the critical target molecules for SNCG's action in breast cancer pathogenesis. Thus, aberrant expression of SNCG stimulates breast cancer growth and progression by enhancing the transcriptional activity of ER-α.
2. Synucleins are emerging as a central player in the fundamental neural processes and in the formation of pathologically insoluble deposits characteristic of Alzheimer's (AD) and Parkinson's (PD) diseases. Most studies of this group of proteins have been directed to the elucidation of their role in the formation of depositions in brain tissue. However, the normal cellular function of this highly conserved synuclein family remains largely unknown. Here we demonstrated that one of the functions of SNCG is activating ER-α signaling. The preventive effect of estrogen on AD has become clear with epidemiological data, suggesting that estrogen may act as a neuroprotectant against the neurodegenerative diseases. The demonstration of ER-α as one of the critical target for SNCG-mediated chaperone activity may indicate a new direction of normal cellular function of synucleins. In this regard, SNCG may be involved in mediating the function of transcriptional activity of ER-α in neuronal cells, thus, the loss or decreased SNCG expression may lower the beneficial effects of estrogen to protect neurons against PD and AD. The potential role of SNCG as a neuroprotectant warrants further investigation.

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